

# **CHEMISTRY**

## 14. Production of Semiquinone by Oxidation of *para*-Monosubstituted Phenols with Singlet Oxygen

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**Keywords:** DMPO, singlet oxygen, semiquinone, phenolic  
compounds, UVA, HPLC

Reactive oxygen species (ROS) involving singlet oxygen ( $^1\text{O}_2$ ) have been implicated both in the aging process and in degenerative disease.  $^1\text{O}_2$  is produced in biological systems by lipid peroxidation and by enzyme reactions involving lactoperoxidase and chloroperoxidase.  $^1\text{O}_2$  is also produced through the interaction of the ultraviolet-A component (UVA) and visible light of sunlight with endogenous photosensitizers such as porphyrins and flavins in the skin.

Phenolic compounds are widely used for the production of pharmaceuticals as well as disinfection, cosmetic and food flavoring goods, and the skin is inevitably exposed to these phenolic compounds. Then, an investigation was done by electron spin resonance (ESR) spectroscopy whether phenolic compounds can react with  $^1\text{O}_2$  to produce the oxidized compounds through the generation of free radical as an intermediate. The phenolic compounds examined were phenol, *o*-methoxyphenol, *p*-cresol, *p*-methoxyphenol, *p*-hydroxyphenylacetic acid, and *p*-hydroxybenzoic acid.

A five-line ESR spectrum was obtained during irradiation with UVA-visible light ( $> 330$  nm) in an air-saturated solution containing 1 mM phenol and 25  $\mu\text{M}$  hematoporphyrin (HP) and assigned to semiquinone (SQ). The signal intensity of SQ was diminished by the addition of a singlet oxygen quencher  $\text{NaN}_3$ , suggesting the participation of  $^1\text{O}_2$  in the production of SQ. The five-line

signal was also observed in other *para*-monosubstituted phenolic compounds such as *p*-methoxyphenol and *p*-hydroxybenzoic acid, but not in *p*-cresol, *p*-hydroxyphenylacetic acid and *o*-methoxyphenol.

The relationship between the signal intensity of SQ and the irradiation time was investigated. In the case of phenol and *p*-hydroxybenzoic acid, the signal intensity increased gradually with the irradiation time. On the other hand, in *p*-methoxyphenol the signal intensity immediately reached the maximum, although it was the smallest value among the three compounds. These results suggest the formation mechanism of SQ in phenol or *p*-hydroxybenzoic acid differs from that in *p*-methoxyphenol.

The production of hydroquinone (HQ) and benzoquinone (BQ) during the reaction of phenol with  $^1\text{O}_2$  was confirmed using high performance liquid chromatography (HPLC)-electrochemical detector (ECD). The time course of the yield of BQ and HQ from the reaction of phenol or *p*-hydroxybenzoic acid with  $^1\text{O}_2$  indicated that BQ is the primary, and HQ the secondary, oxidation product. On the other hand, the increase of equivalent yield of BQ and HQ in *p*-methoxyphenol toward the irradiation time was observed, suggesting that SQ was at first produced, following the production of BQ and HQ by dismutation of SQ. Thus, it is speculated at present that phenol or *p*-hydroxybenzoic acid react with  $^1\text{O}_2$  to form BQ, and the resulting BQ is reduced with HP anion radical to generate SQ. On the other hand, *p*-methoxyphenol reacts with  $^1\text{O}_2$  to form the endoperoxide, and the resulting endoperoxide ultimately undergoes a one-electron reduction by *p*-methoxyphenol to generate SQ.

These results indicate that SQ or BQ are conducted by the oxidation of *para*-monosubstituted phenolic compounds and penetrate into skin with  $^1\text{O}_2$ . The resulting SQ may furthermore lead to the formation of  $\text{O}_2^-$ ,

hydrogen peroxide, and ultimately the hydroxyl radical. Production of ROS can cause aging and carcinogenesis through severe oxidative stress within cells. On the other hand, benzoquinone toxicity develops through the depletion of intracellular biological reductant glutathione.

## 15. DNA Cleavage via Superoxide

### Anion Formed in Photoinduced Electron Transfer from NADH to Cyclodextrin-Bicapped C<sub>60</sub> in an Oxygen-Saturated Aqueous Solution

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**Keywords:** fullerene, DNA cleavage, photodynamic therapy, NADH, cyclodextrin, electron transfer, reactive oxygen species

Fullerenes, such as C<sub>60</sub> and C<sub>70</sub> are sensitive to light at wavelengths longer than 500 nm and thus expected to be an effective photodynamic therapy agent, since bodily tissues are most transparent in this region of wavelengths. Water-soluble  $\beta$ -cyclodextrin-bicapped C<sub>60</sub> (C<sub>60</sub>-CyD) shows an efficient DNA-cleaving activity in the presence of NADH (dihydropyridine adenine dinucleotide) in an O<sub>2</sub>-saturated aqueous solution under visible light irradiation. No DNA cleavage has been observed without NADH under otherwise the same experimental conditions, although singlet oxygen (<sup>1</sup>O<sub>2</sub>) has been detected by the ESR spin-trapping of the C<sub>60</sub>-CyD-O<sub>2</sub> system. This indicates that neither the triplet excited state of C<sub>60</sub>-CyD (<sup>3</sup>C<sub>60</sub><sup>\*</sup>/ -CyD) nor <sup>1</sup>O<sub>2</sub> produced via an energy transfer from <sup>3</sup>C<sub>60</sub><sup>\*</sup>/ -CyD to O<sub>2</sub> is an actual reactive species, which is responsible for the DNA damage under the present experimental conditions. In the presence of NADH, photoinduced electron transfer from NADH to <sup>3</sup>C<sub>60</sub><sup>\*</sup>/ -CyD occurs to yield two equivalents of the radical anion (C<sub>60</sub><sup>•-</sup>/ -CyD), which exhibits its characteristic NIR band at 1080 nm. The dynamics of the photoinduced electron transfer has been examined by monitoring decay of the triplet-triplet absorption band at 740 nm and concomitant rise of the NIR absorption band

at 1080 nm due to C<sub>60</sub><sup>•-</sup>/ -CyD with the use of laser flash photolysis for the C<sub>60</sub>-CyD-NADH system. In the presence of O<sub>2</sub>, C<sub>60</sub><sup>•-</sup>/ -CyD disappears via the electron transfer to O<sub>2</sub> to produce O<sub>2</sub><sup>•-</sup>. An electron transfer from NADH to <sup>1</sup>O<sub>2</sub> also occurs to produce O<sub>2</sub><sup>•-</sup>. The formation of O<sub>2</sub><sup>•-</sup> has been confirmed by the spin trapping with DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide), which is an efficient O<sub>2</sub><sup>•-</sup>-trapping agent. The reorganization energy for the reduction of O<sub>2</sub> to O<sub>2</sub><sup>•-</sup> is evaluated as 4.34 kcal mol<sup>-1</sup>, which agrees with the literature value determined directly for the self exchange between <sup>36</sup>O<sub>2</sub><sup>•-</sup> and <sup>32</sup>O<sub>2</sub>. This indicates that the electron transfer from C<sub>60</sub><sup>•-</sup>/ -CyD to O<sub>2</sub> proceeds via an outer-sphere pathway. The O<sub>2</sub><sup>•-</sup> thus produced gives H<sub>2</sub>O<sub>2</sub>, ultimately yielding hydroxyl radical, which is shown to be the actual DNA-cleaving reagent in this study.

#### Publications:

- 1) Nakanishi, I., Fukuzumi, S., Konishi, T., Ohkubo, K., Fujitsuka, M., Ito, O., Miyata, N. *J. Phys. Chem. B* **106**, 2372–2380, 2002.
- 2) Nakanishi, I., Ohkubo, K., Fujita, S., Fukuzumi, S., Konishi, T., Fujitsuka, M., Ito, O., Miyata, N. *J. Chem. Soc., Perkin Trans. 2* **2002**, 1829–1833.

## 16. Effects of Metal Ions Distinguishing between One-Step Hydrogen- and Electron-Transfer Mechanisms for the Radical-Scavenging Reaction of (+)-Catechin and Vitamin E Model

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**Keywords:** antioxidant, flavonoid, catechin, vitamin E, hydrogen transfer, electron transfer

Catechins contained in green tea are a class of bioflavonoids that show a significant antioxidative activity. Vitamin E (α-tocopherol, α-TOH) is also a very effective biological antioxidant that can scavenge peroxy radicals in biological membranes, preventing oxidative injury by toxic and carcinogenic chemicals. However, little is known about the mechanism of antioxidative radical-scavenging reaction, in which a hydrogen transfer from the phenolic hydroxyl group of catechin or α-TOH to radical species occurs to produce the corresponding phenoxyl radical and the hydrogenated radical species. There are two possibilities in the mechanism of hydrogen-transfer reactions from phenolic antioxidants to radical species, i.e., a one-step hydrogen atom transfer or electron transfer followed by proton transfer. It has previously been demonstrated that the effect of Mg<sup>2+</sup> on the hydrogen-transfer rates from NADH (dihydronicotinamide adenine dinucleotide) analogues to aminoxyl or nitrogen radicals provides a reliable criterion for distinguishing between the one-step hydrogen atom transfer and the electron-transfer mechanisms.

We report herein the effect of metal ions, such as Mg<sup>2+</sup> and Sc<sup>3+</sup>, on the rates of hydrogen transfer from (+)-catechin (**1**) or vitamin E model, 2,2,5,7,8-pentamethyl-6-chromanol (**2**), to radical species, such as 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl

(DPPH<sup>•</sup>), galvinoxyl (G<sup>•</sup>), and cumylperoxyl radicals. The detailed kinetic studies provide valuable mechanistic insight into the antioxidative reactions of natural antioxidants: whether the reaction between natural antioxidants and radical species proceeds via one-step hydrogen atom transfer or via electron transfer.

A kinetic study of a hydrogen-transfer reaction from **1** to G<sup>•</sup> has been performed using UV-vis spectroscopy in the presence of Mg(ClO<sub>4</sub>)<sub>2</sub> in deaerated acetonitrile at 298 K. The rate constants of hydrogen transfer from **1** to G<sup>•</sup> determined from the decay of the absorbance at 428 nm due to G<sup>•</sup> increase significantly with an increase in the concentration of Mg<sup>2+</sup>. The kinetics of hydrogen transfer from **1** to cumylperoxyl radical has also been examined in propionitrile at low temperature with use of ESR. The decay rate of cumylperoxyl radical in the presence of **1** was also accelerated by the presence of Sc(OSO<sub>2</sub>CF<sub>3</sub>)<sub>3</sub>. These results indicate that the radical-scavenging reaction of (+)-catechin proceeds via electron transfer from **1** to oxyl radicals followed by proton transfer rather than via a one-step hydrogen atom transfer. The coordination of metal ions to the one-electron reduced anions of oxyl radicals may stabilize the product, resulting in the acceleration of electron transfer.

In contrast to the radical-scavenging reactions of **1**, no effect of Mg<sup>2+</sup> on rates of hydrogen transfer from **2** to DPPH<sup>•</sup> or G<sup>•</sup> was observed in deaerated acetonitrile at 298 K. Thus, the hydrogen-transfer reaction of **2** proceeds via one-step hydrogen atom transfer rather than via electron transfer followed by proton transfer.

Publications:

- 1) Nakanishi, I., Miyazaki, K., Shimada, T., Ohkubo, K., Urano, S., Ikota, N., Ozawa, T., Fukuzumi, S., and Fukuhara, K.: *J. Phys. Chem. A* **106**, 11123–11126, 2002.
- 2) Nakanishi, I., Fukuhara, K., Shimada, T., Ohkubo, K.,

Iizuka, Y., Inami, K., Mochizuki, M., Urano, S., Itoh, S., Miyata, N., and Fukuzumi, S.: *J. Chem. Soc., Perkin Trans. 2* **2002**, 1520–1524 .

## 17. Enhanced Radical-Scavenging

### Activity of a Planar Catechin

#### Analogue

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**Keywords:** oxidative stress, antioxidant, flavonoid, catechin, hydroxyl radical

Oxidative stress is important in the pathogenesis of neuronal cell death in Alzheimer's and Parkinson's disease. The protective role of antioxidants against such pathogens has been widely studied, and this has promoted the development of antioxidants for the treatment of diseases associated with oxidative stress. Flavonoids such as (+)-catechin (**1**) and quercetin (**2**) are plant phenolic pigment products that act as natural antioxidants (Fig. 14). Quercetin, on one hand, has been shown to protect against oxidant injury and cell death by scavenging free radicals, protecting against lipid peroxidation, and thereby terminating the chain-radical reaction. On the other hand, there have been only a few reports on the use of (+)-catechin for the treatment of free radical-associated disease, whereas the mechanism to scavenge oxygen radical has been well-studied. Furthermore, its ability to scavenge free radicals must be improved, and adequate lipophilicity is needed to penetrate the cell membrane before it is suitable for clinical use. We herein describe the first synthesis and characterization of the antioxidative properties of a planar catechin analogue (**3**) (Fig. 14), in which the catechol and chroman structures in **1** are constrained to be planar.

The planar catechin (**3**) was synthesized via an oxa-Pictet-Spengler reaction using catechin and acetone with  $\text{BF}_3 \cdot \text{Et}_2\text{O}$  as the acid. The structure of **3** was characterized by  $^1\text{H}$  and  $^{13}\text{C}$  NMR and UV-visible spectroscopy. The planar geometry of **3** was

substantiated by single-crystal X-ray crystallography of a tetra-*O*-silylated analogue, in which the four OH groups on the A and B rings are substituted by *t*-Bu(Me)<sub>2</sub>SiO groups.

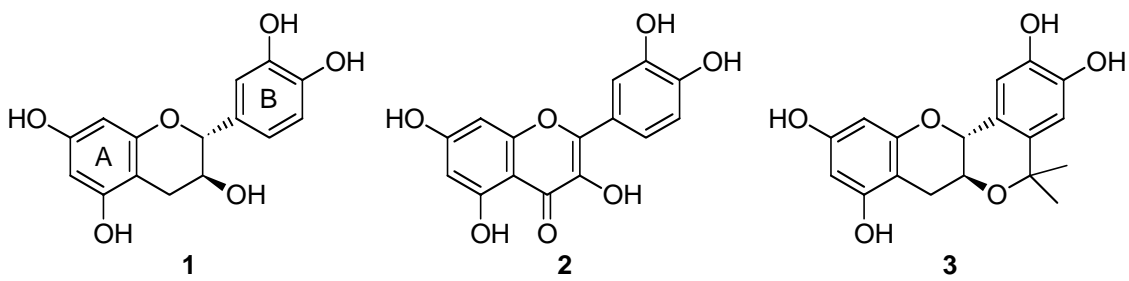
The radical-scavenging activities of **1**, **2**, and **3** were compared using galvinoxyl radical as an oxyl radical species in deaerated acetonitrile at 298 K. From the decay of the absorbance at 428 nm due to galvinoxyl radical with respect to the reaction time were determined the rate constants (*k*) for the radical-scavenging reactions of **1**, **2**, and **3** as  $2.34 \times 10^6$ ,  $1.08 \times 10^6$ , and  $1.12 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ , respectively. Thus, the *k* value for **3** is about 5-fold larger than that for **1** and approximately the same as that for **2**.

Hydroxyl radical is the most reactive among oxygen-derived free radicals responsible for aging and free radical-mediated injury. Therefore, the effects of **1**, **2**, and **3** on hydroxyl radical-mediated DNA breakage were investigated. DNA-strand scission in supercoiled pBR322DNA was induced by a hydroxyl radical-generating system using hydrogen peroxide in the presence of  $\text{Fe}^{3+}$  (Fenton reaction). In contrast to the pro-oxidant effects observed for **1** and **2**, the addition of **3** protected DNA from Fenton reaction-mediated damage and **3** exhibited marked hydroxyl radical-scavenging ability. Since **3** is very lipophilic compared to **1**, the high radical-scavenging ability of **3** might be very useful for suppressing free-radical associated events, especially in the cell membrane.

#### Publication:

Fukuhara, K., Nakanishi, I., Kansui, H., Sugiyama, E., Kimura, M., Shimada, T., Urano, S., Yamaguchi, K., and Miyata, N.: *J. Am. Chem. Soc.* **124**, 5952–5953, 2002.

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**Fig. 14.** Chemical structures of (+)-catechin (1), quercetin (2), and planar catechin (3).

## 18. Scavenging Property of the Indole Derivatives for the Nitrating Intermediate of Peroxynitrite

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**Keywords:** *peroxynitrite, nitrotyrosine, dityrosine, scavenger, tryptamine derivatives, selenium*

Reactive oxygen species are now well-known and important components of the oxidative stress in several diseases, such as inflammation, Parkinson's disease, Alzheimer's disease, etc. It has also been suggested that nitric oxide and its oxidatively activated species, reactive nitrogen species, are involved in the oxidative damage in such diseases. Peroxynitrite (PN) is one reactive nitrogen species, and it has been suggested to be formed from nitric oxide and superoxide *in vivo*. Peroxynitrite is a highly reactive oxidant, and causes nitration on the aromatic ring of free tyrosine and protein tyrosine residues. It was reported that peroxynitrite induced various oxidative damages *in vitro*, for example LDL oxidation, lipid peroxidation, DNA strand breakage and so on. Additionally, tyrosine nitration is assumed to affect the phosphorylation of tyrosine residues in the substrate proteins of tyrosine kinase in the cellular signal transduction. These data imply that the oxidizing and nitrating reactions of peroxynitrite may differently play different pathological roles in the oxidative stress processes. From this point of view, it is very useful and important to discriminate the nitrating (nitrative) damage from the oxidative damage reaction in the peroxynitrite-induced damages.

Various antioxidants have been reported to have an inhibitory effect on the nitration of tyrosine, as well as the

oxidation reaction by peroxynitrite. However, the relationships between those two types of inhibitory effects of single compound have not been quantitatively discussed. Previously we have briefly reported that 5-methoxytryptamine (5MT) and  $\alpha$ -lipoic acid (LA) are selective inhibitors for tyrosine nitration by peroxynitrite, but not for oxidative dityrosine formation (NIRS-37 Annual Report). Adding to this result, we report here the evaluation and comparison of the inhibitory activity for the nitration and the oxidation of tyrosine by peroxynitrite for more than 40 reagents including natural and synthetic compounds, to elucidate the unique property of tryptamine derivatives and one synthetic compound.

Various compounds including natural and synthetic compounds were subjected to the assay for the inhibition of tyrosine nitration and oxidation by peroxynitrite. In the presence of the testing compound, 0.2 mM of freshly prepared peroxynitrite solution was mixed with 1 mM of L-tyrosine in sodium phosphate buffer at physiological pH. The products were analyzed by HPLC with a UV and fluorescence detector system. It was confirmed that 3-nitrotyrosine was formed as a major product of the reaction. The formation of 3-nitrotyrosine was dependent on the concentration of peroxynitrite and L-tyrosine, as previously reported. The formation of 2,2'-dityrosine was also detected by fluorescence at 410 nm (exc. 295 nm). The amounts of 3-nitrotyrosine and 2,2'-dityrosine were quantified as the nitrated and oxidized products, respectively, and the percent production for the control reaction (containing no testing reagents) was calculated. Among the more than 40 compounds showing the inhibitory effect on either nitration or oxidation, the IC<sub>50</sub> values were determined for the 28 compounds having sufficient efficacy.

To compare and characterize the inhibitory effect of the 28 effective compounds, the IC<sub>50</sub> values for the 3-nitrotyrosine formation and the dityrosine formation were plotted on X- and Y-axes, respectively (Fig. 15). We found

that 22 of 28 compounds had values on the line  $y = x$ , meaning that these compounds had the same inhibiting potency for nitration as for oxidation. It was suggested that these compounds scavenged a common intermediate for both nitration and oxidation reactions of peroxynitrite. We also found that the remaining 5 compounds, melatonin, 5-methoxytryptamine, tetrahydro-beta-carboline, tryptophan, lipoic acid and ADCC were off the  $y = x$  line. Melatonin, 5-methoxytryptamine, tetrahydro-beta-carboline, and tryptophan, which all have an indole moiety, showed the inhibitory effect only on the nitration of tyrosine, and they were far above the line. This result indicated that these compounds apparently scavenged different intermediate promoting the nitration reaction specifically. However, ADCC, a selenium-containing synthetic compound, was plotted below the line, indicating that it scavenged the intermediate for the oxidation more effectively than that for nitration.

The product of L-tyrosine and peroxynitrite reaction, 3-nitrotyrosine has a characteristic local absorption maximum ( $\lambda_{max}$ ) at 274 nm. By monitoring the absorbance change at this wavelength, we measured the production rate of 3-nitrotyrosine. L-Tyrosine solution in 0.1 N HCl and peroxynitrite solution in 0.01 N NaOH were mixed, and the absorbance change at 274 nm was recorded with a stopped flow photometer. The reaction rate constant for the tyrosine nitration was found to be  $3.6 \text{--} 3.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  as the second order rate constant. It is known that the reactivity of peroxynitrite for the oxidation is like that of OH radicals, which can react in a diffusion-rate-limiting manner. However, the rate constant for the nitration calculated in this experiment was much smaller than that for OH radical reaction. This result suggests that the nitrating reaction has different rate limiting reaction step(s). From this point of view, the nitrating reaction is not likely to be a direct rebound reaction of caged  $\text{NO}_2$  radicals after electron subtraction by caged OH radicals from the aromatic ring of tyrosine.

Moreover, the pseudo-first order rates are did not exactly linearly correlated with the concentration of tyrosine, so the nitration by peroxynitrite may be a more complicated reaction.

In conclusion, almost all the compounds tested in this study, including typical antioxidants, showed equal inhibitory activity for both nitration and oxidation, suggesting that these compounds scavenged the common or primary intermediate for nitration and oxidation. However, indole derivatives tested here selectively inhibited the tyrosine nitration, suggesting that they scavenged specific intermediates for nitration, probably in later steps of the reaction mechanism. ADCC, a selenium-containing compound, however, inhibited dityrosine formation preferably. We suggested that there might be specific intermediates for tyrosine nitration by peroxynitrite different from the intermediate for the oxidation reaction. The rate-limiting reaction for the nitration was assumed to be very slow compared with OH radical-like reaction of peroxynitrite. It was also demonstrated that the effects of peroxynitrite due to the nitrating reaction could be distinguished from the oxidizing reaction using these specific inhibiting compounds.

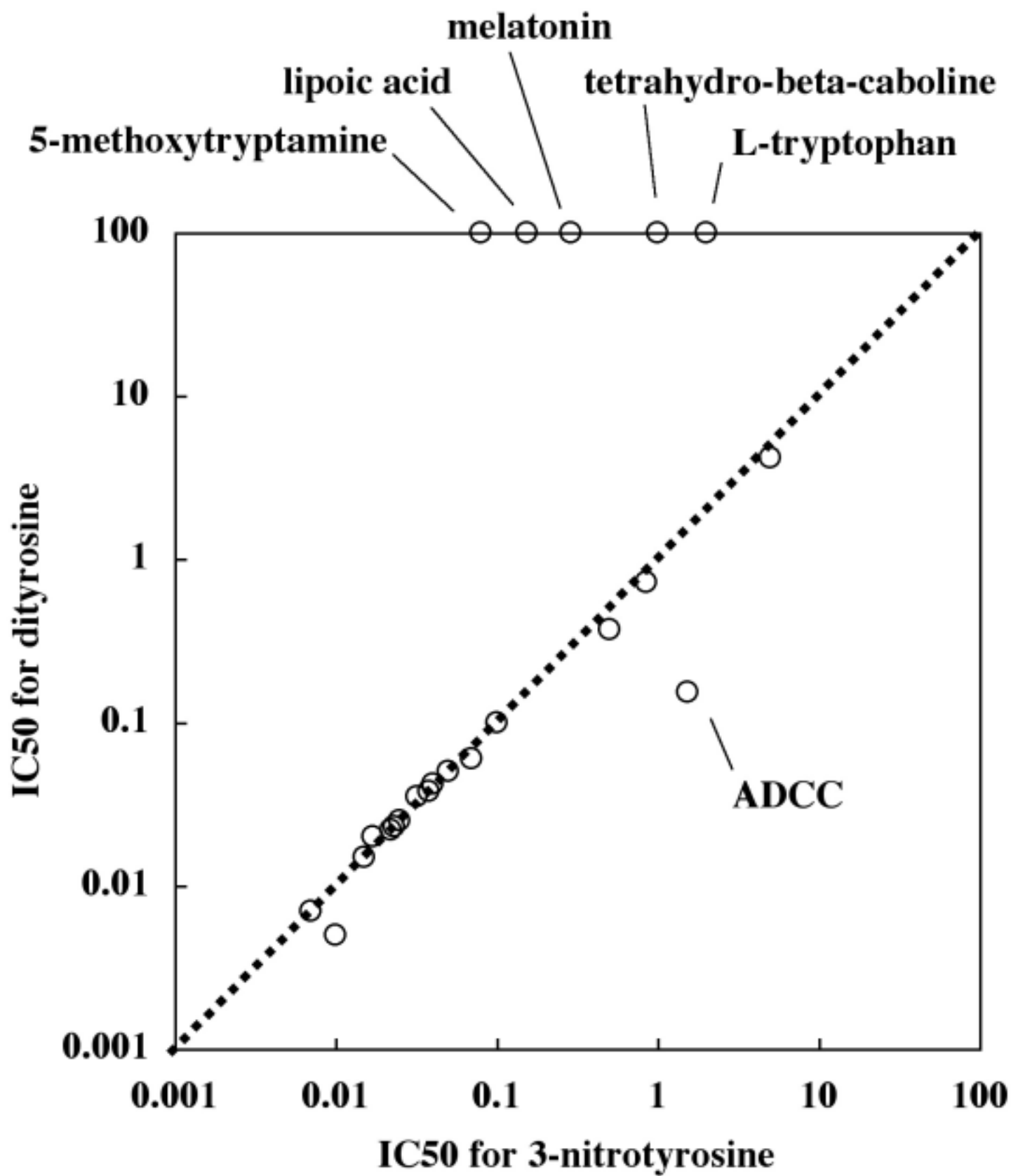


Fig. 15

## 19. Attenuation of the Ability of Cytochrome c for Caspase Cascade Activation due to Protein Nitration by Peroxynitrite

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**Keywords:** *peroxynitrite, cytochrome c, apoptosis, caspase, nitration, tyrosine*

Peroxynitrite, or its equivalent species are very strong oxidants, and candidates for in-vitro substances leading to oxidative and “nitrative stress” in various diseases, such as cardiovascular diseases, brain ischemia, Parkinson’s disease, Alzheimer’s disease, sepsis and so on. Nitration of the free and protein tyrosine residues is a unique reaction of peroxynitrite and its equivalents. The protein tyrosine nitration offers clues that reactive nitrogen species (RNS) like peroxynitrite and its equivalents are produced, and that biological systems are damaged with RNS stresses. There are some reports that the nitration of protein tyrosine residues, including cytochrome c, causes some changes in its functions. It was previously reported that the nitration of single tyrosine residue in cytochrome c by a relatively low dose of peroxynitrite results in the upregulation of its peroxidase activity for hydrogen peroxide, and in the actual impairment of the membrane potential formation, which is important for the ATP synthesis, in isolated mitochondrial preparations (NIRS-4 1 Annual Reports).

It is also well known that cytochrome c is an important player in the mitochondria-dependent apoptotic cell death. Regarding the response for apoptotic stimuli, cytochrome c is released from the intermembrane space to the cytosol, and it forms apoptosome complexes with caspase-9 and Apaf-1 to activate caspase-9 and the downstream caspases, resulting in the apoptic death

execution. Here, we demonstrate that the nitration of cytochrome c by a prolonged exposure to peroxynitrite attenuated its potency for the mitochondria-dependent caspase activation.

For the low-dose repetitive treatment of cytochrome c with peroxynitrite, 1  $\mu$ l of 50 mM peroxynitrite in 0.01 M NaOH was repeatedly added to the solution (1 ml) of 20  $\mu$ M cytochrome c in PBS 20 times at 5-min intervals while mixing (denoted as PN20X50). Because peroxynitrite is unstable at neutral pH, it is practically fully decomposed 5 min after a treatment in PBS. For the control treatment, no peroxynitrite was added to the cytochrome c solution containing decomposed peroxynitrite equivalent to the 20  $\mu$ l of 50 mM peroxynitrite. After the addition of a total of 20  $\mu$ l of peroxynitrite solution, the resultant solution was confirmed to be neutral (pH 7 to 8). All the peroxynitrite-treated cytochrome c solutions were subjected to a gel-filtration with Sephadex G 25, and the concentrations were adjusted according to the absorbance at 409 nm. With the peroxynitrite treatment at the concentration range in this experiment, the maximum absorbance and wavelength of the Soret-band (409 nm) had almost no changes; a blue-shift by only 0.2 nm in wavelength was found. A solution of cytochrome c repetitively treated with low-dose peroxynitrite in the presence of 5-methoxytryptamine (5MT) was also prepared for the experiment on the inhibitory effect of 5MT. Tetranitromethene (TNM)-treated cytochrome c was also prepared. TNM is a well-known protein tyrosine-nitrating reagent. Peroxynitrite-treated cytochrome c was hydrolyzed enzymatically, and analyzed by reversed-phase HPLC, to confirm the nitrotyrosine formation. In the hydrolysate, 3-nitrotyrosine was detected, and it was confirmed that the tyrosine nitration occurred on cytochrome c by a treatment of peroxynitrite.

Caspase activation assay in a cell free system was

carried out using a cytosolic fraction of C6 cells and exogenous peroxynitrite-treated cytochrome c. Peroxynitrite-treated or control cytochrome c was incubated with cytosolic fraction at 30°C for 90 min. The samples were subsequently subjected to SDS-PAGE and immunoblotting with anti-cleaved caspase-3 antibody, and visualized by chemiluminescence with ECL-plus reagents. The cleaved caspase-3 formation indicates the activation of the upstream caspase, caspase-9, meaning that the cytochrome c holds the ability for the caspase cascade activation.

As shown in Fig. 16, the peroxynitrite-treated cytochrome c prepared by repetitive low dose treatments showed a very low activity for the induction of the procaspase-3 cleavage in the *in vitro* apoptosis assay. This attenuation of the caspase cascade activation was not observed when the peroxynitrite-treatment was carried out in the presence of 5MT, which is a nitration selective peroxynitrite scavenger. Furthermore, the TNM-treated cytochrome c also lost its ability for the caspase cascade activation. These results suggested that the nitration on cytochrome c proteins resulted in the attenuation of the potency for the caspase cascade activation. They also implied that the nitrating stress by the low dose and repetitive peroxynitrite exposure, which is likely close to the condition *in vivo*, induced cytochrome c nitration and the suppression of the cytochrome c-dependent caspase cascade activation.

Fig. 16

